CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649062

Inhibitors Influence on Ethanol Fermentation by *Pichia Stipitis*

Augusto S. Silva[a], Antonio M. Oliveira Júnior[a],Carlos Eduardo de Farias Silva[b], Ana Karla S. Abud*[a]

[a]Food Technology Department, Federal University of Sergipe, São Cristóvão, Sergipe, Brazil
[b]Department of Industrial Engineering DII, University of Padova, Padova, Italy
ana.abud@gmail.com

Considered as a promising alternative energy source, agro-industrial biomass needs a pre-treatment step for conversion to sugars, which can release not only hexoses but also pentoses and other sugars, as well as inhibitors. The inhibitory effect of acetic acid, furfural and hydroxymethylfurfural in ethanol kinetics production from *Pichia stipitis* yeast, in the presence of commercial glucose and xylose and Yeast Nitrogen Base (YNB) is investigated in this work. Fermentations took place in 250 mL Erlenmeyer flasks, at room temperature, 150 rpm and 72 h without pH control, with an initial concentration of 10^9 cells/mL. The mixture of the inhibitors negatively influence the process, not observing cell growth, and about 95% of non-viable cells at 48 h of fermentation, despite the consumption of 40% glucose and 35% xylose. In comparison with the control test (without addition of inhibitors), the isolated use of 0.05 g/L hydroxymethylfurfural and 0.25 g/L furfural presented profile slightly lower, with almost complete conversion of xylose and 60% glucose to ethanol, reaching ethanol yield around 40%. Isolated addition of 1.75 g/L acetic acid significantly influenced the process, leading to yield less than 5% and 80% of non-viable cells at 48 h, indicating that this is the main inhibitor of ethanol production.

1. Introduction

The global threat warming, along with depletion of crude oil at world level and increasing energy demand, imply the urgent need to replace fossil fuels by green biofuels. Fuels from biomass, such as bioethanol, provide a promising alternative since its energy is already included in the global carbon cycle, which entails a significant reduction in carbon dioxide (Karagöz et al., 2012). Among raw materials biomass, lignocellulosic residues offer attractive renewable sources for the bioethanol production, as they do not compete with the food industry and represent the most abundant carbohydrate reserves in the world (Saha et al., 2013).
The lignocellulosic materials are an abundant source of renewable energy worldwide. Representing 90% of the dry matter, these complex carbohydrates polymers are composed primarily by cellulose $(C_6H_{10}O_5)_x$, hemicellulose $(C_5H_8O_4)_m$ and lignin $(C_9H_{10}O_3(OCH_3)_{0.9\text{-}1.7})_n$, with the remainder (10%) of extractives and ash (Balat, 2011). This structural complexity, defined as recalcitrance, restricts microbial and enzymatic accessibility (Pu et al., 2013), making characterization of biomass a crucial factor in the production of biofuel during the bioconversion process. Hendriks (2009) reports lignin content, cellulose crystallinity and particle size as the main factors that limit the digestibility of the hemicelluloses and cellulose present in the lignocellulosic biomass.
Hemicellulose, along with cellulose and lignin, can be easily hydrolyzed to monomeric sugars under process mild conditions (Silva, 2012). Appropriate pretreatments, based on the characteristics of each raw material, should be applied (Karagöz et al., 2012) to deconstruct biomass, which can include physical pretreatments (downsizing), physico-chemical pretreatments (liquid hot water, steam explosion, ammonia fiber explosion), chemical pretreatments (acids, alkaline, oxidative alkaline, wet oxidation, ozonolysis) and biological pretreatments (Talebnia et al., 2010).
No single microorganism can efficiently convert the sugars to ethanol. Individually, *Saccharomyces cerevisiae* and *Zymomonas mobilis* are effective for the conversion of glucose, while *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* are efficient for xylose conversion (Fu; Peiris, 2008).

Please cite this article as: Silva De Souza A., Oliveira Junior A.M., De Farias Silva C.E., Abud De Souza A.K., 2016, Inhibitors influence on ethanol fermentation by pichia stipitis, Chemical Engineering Transactions, 49, 367-372 DOI: 10.3303/CET1649062

Among the fermenting pentoses microorganisms, *Pichia stipitis* appears as capable of fermenting xylose and other important hexoses to ethanol from hydrolyzed lignocellulosic biomass, with conditions as pH, temperature, oxygen, agitation and medium composition as important factors in the process of bioconversion (Sunitha et al., 1999; Nigan, 2001; Cabral et al., 2005; Agbogbo et al., 2008; Farias et al., 2013.). *Pichia stipitis* also does not require the addition of vitamins to the fermentation of xylose and is able to use a wide range of sugars as a substrate, such as glucose and cellobiose (Agbogbo; Wenger, 2007; Bellido et al., 2011). This paper discusses the influence of the main inhibitory compounds formed in lignocellulosic biomass pretreatment, acetic acid, furfural and hydroxymethylfurfural, isolated or in mixture, in the ethanol fermentation process with the yeast *Pichia stipitis* on a minimal medium containing commercial xylose and glucose as a carbohydrate sources.

2. Materials and Methods

Pichia stipitis NRRL Y-7124 was gently conceed by Embrapa Agroenergia. The yeast was maintained on YPX agar tubes (20 g/L yeast extract, 10 g/L peptone, 20 g/L xylose and 20 g/L agar) at 4 °C in a refrigeration chamber.

The culture medium was composed of 20 g/L xylose, 3 g/L glucose and 6.7 g/L YNB (Yeast Nitrogen Base). The pH was adjusted to 4.5, optimal condition for the yeast growth, and, then, sterilized at 121 ºC and 1 atm for 15 min in autoclave. Inoculum was grown on a rotator shaker at 150 rpm and 30 °C for 24 h (exponential growth phase). The cells were counted in a Neubauer chamber to determine the volume need to adjust 2.10^7 cells/mL as initial concentration in all experiments. The cells were recovered by centrifugation (3500 rpm, 10 min) and resuspended in the fermentation medium.

The fermentation medium was composed of 20 g/L xylose, 3 g/L glucose and 6.7 g/L YNB (Yeast Nitrogen Base). The inhibitors compounds were added in predefined amounts concentrations [acetic acid (1.75 g/L), furfural (0.25 g/L) and hydroxymethylfurfural (0.05 g/L)], according to literature (Bellido et al., 2011) and experimental data with sugarcane bagasse pretreatments. The pH was adjusted to 4.5 with hydrochloric acid solution 2 N and the medium was sterilized at 121 ºC and 1 atm for 15 min in autoclave. Fermentation processes were carried out in 250 mL Erlenmeyer flasks containing 100 mL of culture medium at room temperature and 150 rpm for 72 h, without pH control and under microaerobic conditions (flasks closed with cotton plug). During the experiments, samples were taken every 24 h for pH measurement, determination of cell viability, cell growth, glucose and xylose consumptions and ethanol production. The fermentation without addition of inhibitors (control) and blends were performed in triplicate.

Cellular growth (X) was determined by measuring optical density of cells at 600 nm and correlated with dry weight. The samples were centrifuged at 3500 rpm for 10 min before the measurements of xylose, glucose and ethanol concentrations. Total sugar concentration was determined by the colorimetric method of the dinitrosalicylic acid (DNS), after boiling for 5 min and read at 540 nm (Miller, 1959), which was correlated with a calibration curve. Glucose was determined by a Liquiform kit (Labtest), at 505 nm. Xylose was obtained by the difference between total sugar and glucose concentration. Ethanol analysis was carried out by simple distillation method and determined by the spectrophotometric potassium dichromate method (Joslyn, 1970), with basis on a standard curve with predefined ethanol concentrations also subjected to distillation process. The measurements were performed in triplicate. The percentage of non-viable cells was determined by a specified volume of the fermentation broth mixed with Methylene Blue solution, counting the cells in a Neubauer chamber. Yeast cells stained blue were considered dead (non-viable), while it remained colorless alive (viable).

Ethanol yield factor ($Y_{P/S}$ g/g) was defined as the ratio between ethanol concentration (ΔP, g/L) and substrate xylose consumed (ΔS, g/L). Cells yield factor ($Y_{X/S}$, g/g) was defined as the ratio between cells formed (ΔX, g/L) and substrate xylose consumed (ΔS, g/L). Ethanol volumetric productivity (Q_P, g/L.h) was calculated as the ratio between the maximum ethanol concentration (P, g/L) and the respective fermentation time (h). The sugar conversion efficiency in ethanol (η,%) was determined as the ratio of YE / S (g / g) and the theoretical value (0.511 g / g) of parameter present (Hahn-Hägerdal et al., 1994).

3. Results and Discussion

The kinetic fermentation profiles and cell viability for these studies are shown in Figure 1. It should be noted that *Pichia stipitis* proved to be suitable for development in isolated presence of inhibitors hydroxymethylfurfural (HMF) and furfural (F), being the HMF assay the one with the best performance, not influencing cell growth and substrate consumption, with a similar profile to rehearsals without the presence of inhibitors (C). In contrast, tests with addition of inhibitor mixture (M) and isolated acetic acid (HAc) indicated a

positive effect on inhibition of acetic acid (HAc) and a mixture of inhibitors (M) did not reach 0.50 g/L of cell concentration throughout the process.

Compared to previous studies with only xylose as carbon source, the use of another source of energy (glucose) to experiments C, F and HMF increased the microorganism cell growth, resulting in a double cell concentration after 48 h fermentation. Due to this growth rate, it was already observed growth decline at the nesxt point assessed (72 h). The control test (C) present better results, with about 2.50 g/L in growth log phase, followed by HMF and F, respectively. These results corroborate the study Bellido et al. (2011), who evaluated the same inhibitors in fermentation in rich medium and presence of xylose and glucose.

Initially, the fermentation pH of C, F and HMF processes were 5.0 but, after 24 hours this value decreased to 3.0, remaining there until the end of the studies for these assays. From this time proved to be an increase in cell and ethanol production production, while in the experiments M and HAc the pH value of 4.0 was maintened throughout all the experiment. Du Prezz (1994) reported that the yield of ethanol by yeast *Pichia stipitis* was greatly influenced by the pH variation between 2.5 and 5.5, being the optimum pH between 4.0 and 5.5. It is noteworthy that there may be variations between the values or optimum pH ranges due to different fermentation media used, cultivation conditions or different strains.

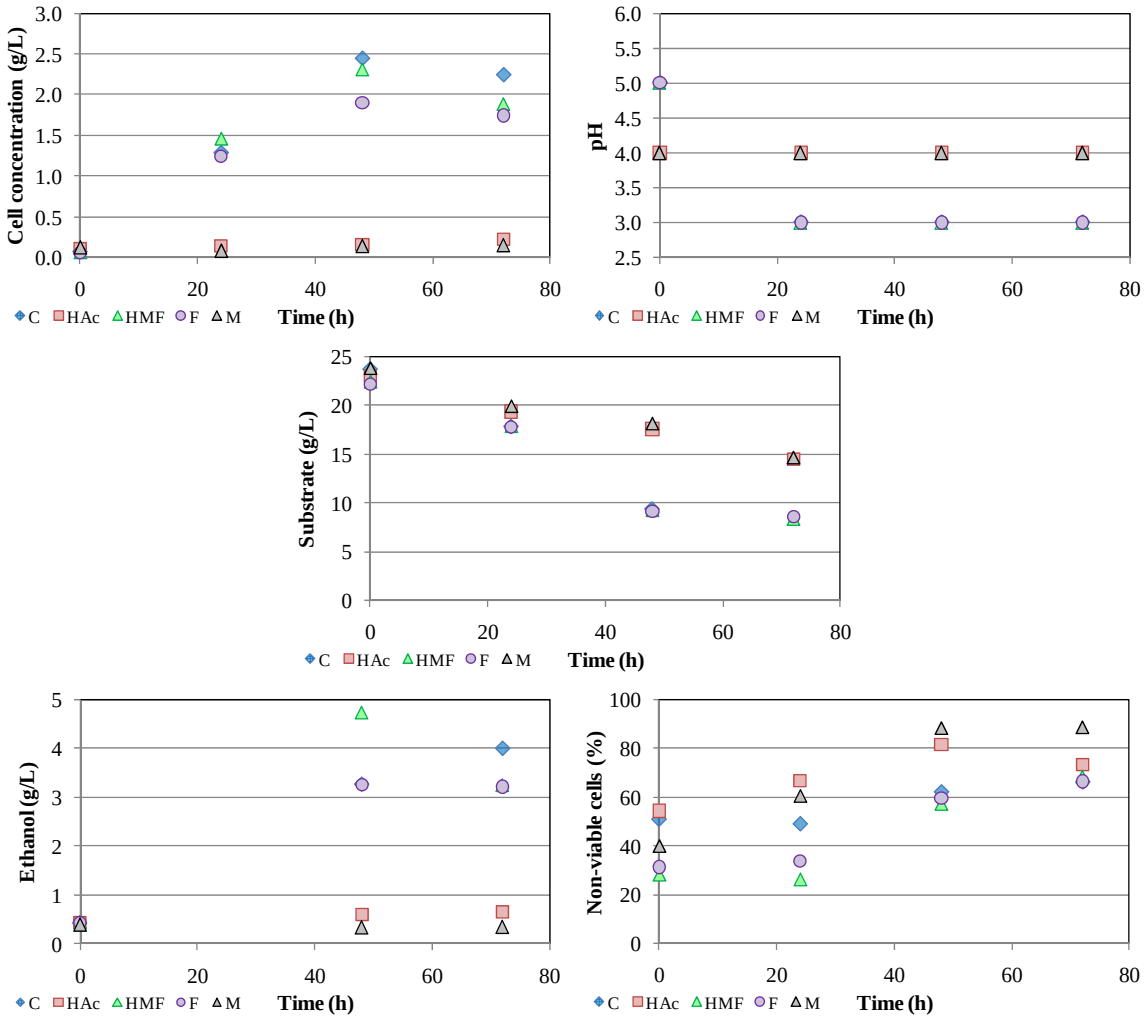

Figure 1: Kinetic behavior of the fermentation of glucose and xylose with variables: (a) cell concentration, (b) pH, (c) substrate consumption, (d) ethanol production and (e) percentual of non-viable cells.

The substrates present in these studies were analyzed individually and in total, so that it was possible to evaluate the influence of consumption when the another energy source addition in the process. The total substrate after 24 h had the biggest decline for C testing, HMF and F, resulting in less than 10 g/L at the end

of the experiment. In contrast, M and HAc had lower substrate consumption rates during the entire course of the experiment, as shown in Figure 1.

Ethanol production shows linear growth until the last point of the study, yielding 4 g/L ethanol, whereas HMF generated 4.5 g/L at 48 h, with a later drop in production at 72 h, while the maximum production for F occurred at 48 h, keeping constant to the end. The experiments with M and HAc showed low values for ethanol production, obtaining the maximum point in the last analysis (72 h) with 0.66 g/L and 0.35 g/L, respectively.

The percentage of non-viable cells also makes it possible to analyze cell growth of fermentation trials. Initially, control experiments (C) and acetic acid (HAc) had a rate of approximately 50% of non-viable cells, with the HAc increased in the following days as well as the mixture (M), that the time of 48 h had about 80% of its dead cells. Tests with HMF and F had the lowest initial percentages, but in time 48 h, similarly to C, had 60% of non-viable cells, increasing in the next time, which demonstrate the detrimental effect of the inhibitors for these tests. According to Ferreira et al. (2011), an elevate initial cell concentration will consist in a higher number of viable cells for the biomass and product formation, being, consequently, a strategy to ameliorate the toxic effect of inhibitor compounds.

The consumption of xylose and glucose alone occurred in different ways (Figure 2), where the glucose was consumed almost completely in tests C, HMF and F, having concentration of from 0.16 to 0.12 g/L at the end of the experiment. The same tests showed half of the starting xylose concentration used (20 g/L) in the process in 72 hours of interval, while in other tests (HAc and M) consumption of the two substrates are made more slowly, not having low values after the entire fermentation process. For glucose consumption, the inhibitors mixture (M) showed constant over time, different from HAc that had an increasing decline during the same period (Figure 2). These results differed from the reports of some authors, as Nigam (2001) and Bellido et al. (2011), which in their work glucose was completely consumed before xylose, with the use of a higher concentration of the same substrates.

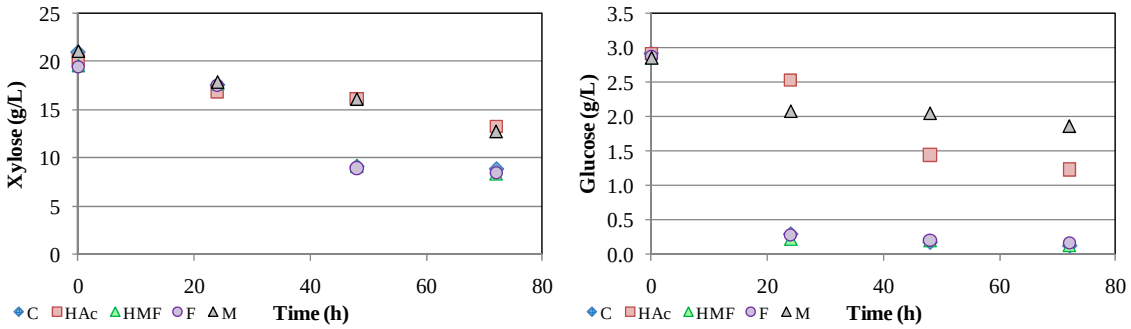

Figure 2: Consumption of xylose (a) and glucose (b) in function of fermentation time.

The presence of glucose, a readily metabolisable hexose can induce regulatory problems due to the interaction between the metabolism of different sugars, resulting in poor uptake of xylose and thus influence the yield and productivity (Tavares et al., 2000).

The percentage of substrate consumed throughout the fermentation process was also obtained to facilitate studies of this behavior, as shown in Figure 3, where one can see that almost 100% of the glucose was used in C tests, HMF and F, followed by HAc (60%) and M (35%). The xylose had consumed more than 50% for C, F and HMF and near 40% for HAc and M.

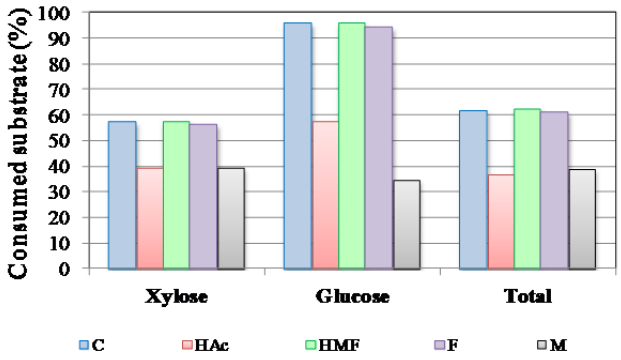

Figure 3: Percentages of the substrate consumption in fermentation processes with xylose and glucose

Aloisio et al. (2014) report the ethanolic fermentation by *P. stipitis* in a glucose/xylose mixture obtained from mulberry treatments, where after 24 h the yeast was able to consume all glucose but only the 11% of xylose, producing 2,6 g/L of ethanol. In the synthetic culture media applied, the experiments without inhibitors (C) and isolated presence of furfural (F) and hydroxymethylfurfural (HMF) have a consume of practically all glucose and 56% of xylose, producing 3.59, 2.79 and 2.81 g/L of ethanol, respectively.
Table 2 shows the changes in cell growth (ΔX), substrate consumption (ΔS) and production of ethanol (ΔP) after 72 h of fermentation and the values found for the μmax parameters $Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$, P_X, P_Q and η obtained for each experiment.

Table 1: Kinetic parameters and conversion from tests with xylose and glucose.

	ΔX (g/L)	ΔS (g/L)	ΔP (g/L)	μ (h^{-1})	Xylose consumed (%)	$Y_{X/S}$ (g/g)	$Y_{P/X}$ (g/g)	$Y_{P/S}$ (g/g)	P_Q (g/L.h)	P_X (g/L.h)	η (%)
C	2.19	14.67	3.59	0.050	57.5	0.149	1.639	0.245	0.050	0.030	47.89
Hac	0.11	8.44	0.23	0.009	39.3	0.013	2.091	0.027	0.003	0.002	5.33
HMF	1.39	14.04	2.81	0.047	57.6	0.099	2.022	0.200	0.039	0.019	39.17
F	1.69	13.53	2.79	0.048	56.5	0.125	1.651	0.206	0.039	0.023	40.35
M	0.02	9.25	0.00	0.003	39.4	0.002	0.000	0.000	0.000	0.000	0.00

The fermentation processes C, F and HMF, obtained total substrate conversion cells ($Y_{X/S}$) next to 0.15 g/g found by Roberto et al. (1994). Similar values were also found for the conversion of only xylose for these assays 0,182 g/g, 0.123 g/g and 0.154 g/g, respectively, whereas the conversion of glucose into cells values have been elevated to 0,785 g/g, 0.509 g/g and 0.624 g/g for the same tests, fact that can be attributed to consumption and assimilation of this carbon source more easily than xylose.
The conversion of substrate in ethanol ($Y_{P/S}$) resulted in 0.245, 0.200 and 0.206 g/g for assays C, HMF and F, respectively, values close to those found by Cabral et al. (2005), who obtained 0.34 to 0.37 g/g in similar studies, in pH range (4.0 to 6.0) and rich fermentation medium, without the presence of inhibitors. Nigam (2001), at pH 6.5, achieved 0.41 g/g. The fermentation with HAc obtained 0.027 g/g for the same parameter, much lower than those found by Bellido et al. (2011) using the similar concentration of acetic acid (1.5 g/L), reaching 0.44 g/g. In the test without the addition of inhibitors (C) the yield of ethanol conversion was 1,639 g/g.
The values of the maximum specific growth rate (μ_{max}) were lower than those found by Farias et al. (2013), approximately 0.050 h^{-1} to the fermentations without inhibitors (C), HMF and F and below 0,010 h^{-1} for HAc and M. This may be due to non-aeration means.
The ethanol productivity (Q_P) was also evaluated, showing low ethanol production for all tests, presenting values of 0.050 g/L.h for C fermentation and 0.039 g/ L.h for F and HMF assays, which does not corroborate with those found by Silva et al. (2011) using only xylose and different concentrations, that reached 0.34 g/L.h, and Ferreira et al. (2011), which obtained 0.13 g/L.h in the fermentation with 30 g/L xylose. Similar values were found by Nigam (2001), using 1.5 g/L of furfural on inhibition studies, obtaining 0.07 g/L.h. The HAc experiment showed values lower than that reported by Bellido et al. (2011), applying 1.5 g/L of acetic acid and obtaining, after 168 h of fermentation, 0.10 g/L.h.
Isolated addition of HMF and F, compared to the assay without the presence of inhibitors (C), presented ethanol yields 18.2 and 15.7% lower, respectively, while the addition of acetic acid (HAc) inhibited almost completely (90%) the ethanol production.
The results indicate the need to control the concentration of these inhibitors in the extract resulting from the lignocellulosic biomass pretreatment, in particular the chemical pretreatment where a greater amount of these components is generated, in order to improve ethanolic fermentation of pentoses by *Pichia stipitis* yeast.

4. Conclusions

The yeast *Pichia stipitis* proved capable of growing in the presence of the isolated main inhibitors of the pre-treatment of lignocellulosic biomass, but showed that the mixture of these components (M) is able to completely inhibit the fermentation process. The use of other carbon source (glucose) can drive positively all parameters and variables studied kinetics, even though their use makes it difficult to assimilate xylose by *Pichia stipitis*. The presence of hydroxymethylfurfural (HMF) and furfural (F) in a concentration of 0.05 g/L and 0.25 g/L, respectively, showed a positive effect on ethanol fermentation, in particular with only HMF, which showed values of cell growth and consumption of similar substrate the control assay (C).
It is evident that the bioconversion of pentoses (xylose) to ethanol is a process influenced by several factors such as growing conditions, type of fermentation, strain used, pH, temperature, initial concentration of viable

cells and substrate tolerance of ethanol yeast, aeration, among others, which should be studied and analyzed in order to obtain more detailed findings about the kinetic process.

Acknowledgments

The authors gratefully acknowledge the partnership and support from the Embrapa Agroenergia.

Reference

Agbogbo F.K., Haagensen F.D., Milam D., Wenger K.S., 2008, Fermentation of acid pretreated corn stover to ethanol without detoxification using *Pichia stipitis*, Applied Biochemistry and Biotechnology 145, 53-58.

Agbogbo F.K., Wenger K.S., 2007, Production of ethanol from corn stover hemicellulose hydrolyzate using *Pichia stipitis*, Journal of Industrial Microbiology and Biotechnology 34, 723-727.

Aloisio L., De Santis A., Spera D.M., Pignatelli V., Albergo R., 2014, Innovative of second generation ethanol production from biomass crops by *Pichia stipitis*, Chemical Engineering Transactions 38, 115-120.

Balat M., 2011, Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review, Energy Conversion and Mangement 52(2), 858-875.

Bellido C., Bolado S., Coca M., Lucas S., González-Benito G., García-Cubero M.T., 2011, Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*, Bioresource Technology 102, 10868-10874.

Cabral J.C.A., Silva J.P.A., Roberto I.C., 2005, pH influence in ethanol production by Pichia stipitis, In: 9º Encontro Latino Americano de Pós-Graduação, Lorena-SP (in Portuguese).

Du Preez J.C., 1994, Process parameters and environmental factors affecting-xylose fermentation by yeasts, Enzyme and Microbial Technology 16, 944-956.

Farias D., Andrade R. R., Maugeri Filho F., 2013, Kinetic modeling of ethanol production by *Scheffersomyces stipitis* from xylose, Applied Biochemistry and Biotechnology 172, 361-379.

Ferreira A.D., Mussato S.I., Cadete R.N., Rosa C.A., Silva S.S., 2011, Ethanol production by a new pentose-fermenting yeast strain, *Scheffersmoyces* UFMG-IMH 43.2, isolated from the Brazilian forest, Yeast 28, 547-554.

Fu N., Peiris P., 2008, Co-fermentation of a mixture of glucose and xylose to ethanol by *Zymomonas mobilis* and *Pachysolen tannophilus*, *World J Microbiol Biotechnol*. 24, 1091-1097.

Hahn-Hägerdal B., Jeppsson H., Skoog K., Prior B.A., 1994, Biochemistry and physiology of xylose fermentation by yeasts, Enzyme and Microbial Technology 16, 933-943.

Hendriks A.T.W.M., Zeeman G., 2009, Pretreatments to enhance the digestibility of lignocellulosic biomass, Bioresource Technology 100, 10-18.

Karagoz P., Rocha I.V., Ozkan M., Angelidaki I., 2012. Alkaline peroxide pretreatment of rapeseed straw for enhancing bioethanol production by same vessel saccharification and Co-fermentation. Bioresour. Technol. 104, 349–357.

Miller G.L., 1959, Use of dinitrosalicilic acid reagent for determination of reducing sugar, Analytical Chemistry 31(3), 426-428.

Nigam J.N., 2001, Ethanol production from wheat straw hemicellulose hydrolysate by *Pichia stipitis,* Journal of Biotechnology 87, 17-27.

Pu Y., Hu F., Davison B.H., Ragauskas A., 2013, Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments, Biotechnology for Biofuels 6(15), 1-13.

Roberto I.C., Lacis L.S., Barbosa, M.F.S., Mancilha, I.M., 1994, Utilization of sugar cane bagasse hemicellulosic hydrolysate by Pichia stipitis for the production of ethanol. Process Biochemistry 26(1), 15-21.

Silva F.V., 2012, Panorama e perspectivas do etanol lignocelulósico, Revista Liberato 13(20), 43-58.

Silva J.P.A., Mussatto S.I., Roberto I.C., Teixeira J.A., 2011, Ethanol production from xylose by *Pichia stipitis* NRRL Y-7124 in a stirred tank bioreactor, Brazilian Journal of Chemical Engineering 28(1), 151-156.

Saha B.C., Yoshida T., Cotta M.A., Sonomoto K., 2013, Hydrothermal pretreatment and enzymatic saccharification of corn stover for efficient ethanol production, Ind. Crop. Prod. 44, 367–372.

Sunitha K., Lee J.K., Oh T.K., 1999, Optimization of medium components for phytase production by *E. coli* using response surface methodology**,** Bioprocess Engineering 21, 477-481.

Talebnia F., Karakashev D., Angelidaki I., 2010, Production of bioethanol from wheat straw: an overview on pretreatment, hydrolysis and fermentation, Bioresour. Technol. 101, 4744–4753.

Tavares J.M., Duarte L.C., Ammaral-Collaço M.T., Gírio F.M., 2000, The influence of hexoses addition on the fermentation of D-xylose in *Debaryomyces hansenii* under continuous cultivation, Enzyme and Microbial Techonology 26, 743-747.